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Fluorescence Staining of Laryngeal Neoplasms After Topical Application of 5-Aminolevulinic Acid: Preliminary Results

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Background and Objective: **The prognosis of patients suffering from laryngeal carcinomas can be improved by early diagnosis. Exact demarcation of tumor margins could contribute to an optimum preservation of the larynx. Therefore, the aim of the present study was the evaluation of 5-aminolevulinic acid (5-ALA)-induced protoporphyrin IX (PPIX) fluorescence as a new diagnostic procedure for the detection of laryngeal cancer.**
Study Design/Materials and Methods: **Sixteen patients with suspected malignancies of the larynx received 0.6 wt% 5-ALA-NaCl solution by means of a medical nebulizer. After a period of 1–2 hours, the patients underwent microlaryngoscopy under white light and fluorescence illumination (λ_{ex} = 375–440 nm). A quantitative analysis of the fluorescence contrast between neoplastic and surrounding tissue was performed using an optical multichannel analyzer.**
Results: **Carcinoma, carcinoma in situ, and dysplasia showed red fluorescence that could be attributed to the 5-ALA-induced formation of PPIX. The surrounding normal tissue exhibited autofluorescence in the green spectral range, which was greatly reduced within the tumor. The results of macroscopic red fluorescence staining were correlated with the histologic diagnosis.**
Conclusion: **According to these preliminary results, the presented method seems to be a promising adjunct diagnostic procedure for the early identification of malignant neoplasms in the larynx. The aim of further investigations is the assessment of sensitivity and specificity and an evaluation of fluorescence-guided laser resections of laryngeal cancer. Lasers Med. Surg. 25:414–420, 1999.** **© 1999 Wiley-Liss, Inc.**

Key words: 5-aminolevulinic acid; inhalation; laryngeal neoplasms; protoporphyrin; spectroscopy

INTRODUCTION

In Germany almost 1,700 patients died from laryngeal cancer (ICD 161) in 1990, thus accounting for a mortality rate of 2.1 [1]. There are an estimated 3,200 new cases annually in men and 500 new cases in women. The reported 5-year sur-

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vival rates for all stages range from 65.4% for men to 75.8% for women [1]. A recent U.S. study has pointed out that survival rates are significantly higher for early stage carcinomas. However, increased efforts at patient and physician education aimed at early detection did not seem to have an impact [2].

Although macroscopic aspects suggest a malignancy of the larynx, it may be impossible to diagnose the tumor with the first biopsy [3]. New techniques for tumor detection could allow guided biopsies that reduce the risk of nonrepresentative histologic samples and help avoid delays in diagnosis and, hence, therapy. In addition, exact demarcation of tumor margins could help to preserve the organ by enabling the surgeon to select narrow resection margins.

A variety of methods using supravital staining, tissue autofluorescence, or fluorescent tumor markers has been investigated in past decades to improve the diagnosis of laryngeal malignancies.

Several researchers have evaluated toluidine blue staining for the detection of malignant glottic lesions [4–8]. Whereas Thomsen and Thomsen [8] reported insufficient reliability in the detection of carcinomas in situ, Lundgren et al. [5] observed an overall sensitivity of 91% and specificity of 52% with this method (n = 202). Toluidine blue was manually applied to the vocal cords with a cotton applicator after removal of mucus [5,8].

Fryen et al. [9] performed in vitro and in vivo studies using autofluorescence (excited at 365 nm) for the detection of malignancies of the oropharynx, hypopharynx, and larynx. They were not able to observe a distinct difference between fluorescence intensity in the tumor and adjacent epithelium in vitro. However, in vivo, an intense fluorescence of tumor borders could be observed, whereas the malignant areas seemed to show slightly weaker fluorescence than normal tissue. They concluded that, because of the multifactorial genesis of fluorescence, a great deal of experience is required for the interpretation of the findings obtained by this method.

An autofluorescence imaging system (LIFE, Xillix, Vancouver, Canada), originally developed for the detection of lesions in the tracheobronchial tree, was evaluated for the detection of laryngeal lesions [10,11]. Although it was possible to identify some lesions that appeared innocuous under white-light illumination, difficulties were encountered in the presence of keratinization and bleeding. Zargi et al. [11] found the appearance of cancer to be variable, so that considerable experience was necessary to recognize malignancies. The sensitivity of this procedure was 82% and the specificity was 76%.

Another fluorescence-based approach to tumor detection is the administration of exogenous fluorophores that selectively accumulate in malignant tissues. Leonhard et al. found that, after intravenous administration of hematoporphyrin derivatives (HpD), a strong red fluorescence could be observed in malignant neoplasms of the larynx [12,13]. The reliability of this method seemed to be very good. However, systemic administration of HpD has a photosensitizing effect on the entire skin. Thus, a major drawback of the method was that patients had to be urged to avoid direct exposure to sunlight for 10 days.

Tetracycline-induced fluorescence was evaluated as a diagnostic tool for laryngeal, pharyngeal, and oral lesions by Dunn and Devine [14]. It showed a specificity of 100% and a sensitivity of 89%. Nonetheless, they were not able to obtain a clear-cut demarcation of the lesions examined. Furthermore, the technique proved to be unsuitable for clinical use because of the complex equipment needed and the time-consuming procedure.

A new method for the staining of malignant tissues is the application of 5-aminolevulinic acid (5-ALA), which selectively induces the accumulation of protoporphyrin IX (PPIX) fluorescence in tumors. This technique has been reported to be a powerful tool for the detection of malignancies located in the urinary bladder [15–17], brain [18], and oral cavity [19,20]. It was found to be easy to use and free of side effects when topically applied [21].

Therefore, the aim of the present study was to evaluate 5-ALA-induced PPIX fluorescence for the detection of laryngeal neoplasms.

MATERIALS AND METHODS

5-Aminolevulinic Acid

5-ALA was topically applied to the patient's larynx by inhalation at rapid pace with intermittent vocalization. For this purpose, 30 mg 5-ALA hydrochloride (Medac, Hamburg, Germany) was dissolved in 5 ml 0.9% NaCl solution. This solution was nebulized with a Pari nebulizer (Pari, Starnberg, Germany). The average droplet size was 9 μm.

Patients

Sixteen patients with suspected or histologically proven malignancies of the larynx were investigated. Informed consent was obtained from all patients. 5-ALA was applied 1–2 hours before microlaryngoscopy.

Fluorescence Imaging

The examination was performed through an optimized endoscope (Hopkins 0°, Art. No. KSTEXB001-3 or 27005AI, Storz, Tuttlingen, Germany) designed to minimize the loss of light intensity. For fluorescence excitation, a xenon short arc lamp equipped with a special filter system (D-Light, Art. No. 20133201, Storz) was used. A footswitch allowed changing between white-light illumination and fluorescence excitation light (λ_{ex} = 375–440 nm).

To allow fluorescence imaging, most of the remitted excitation light was blocked by the integrated filter of the endoscope (long pass = 440 nm). A total blocking of the remitted excitation light could be achieved with an additional filter (OG515, Schott, Mainz, Germany) attached to the eyepiece of the endoscope. Video images were obtained by using a target-integrating, high-resolution color charge coupled device (CCD) camera (Telecam SL PAL, Art. No. 20212020, Storz) mounted on the endoscope. White-light and fluorescence findings were recorded for documentation on video tape (S-VHS, Panasonic, Osaka, Japan).

Fluorescence Spectroscopy

In addition to the fluorescence imaging, spectroscopic measurements were performed on nine patients to quantify the fluorescence findings. Fluorescence spectra were taken from neoplastic lesions, corresponding normal tissue, and a reference target.

For spectral measurements, an optical multichannel analyzer (S2000, OceanOptics, Dunedin, FL) was attached to the endoscope rather than the CCD camera. A beam splitter provided the surgeon with a fluorescence image of sufficient quality to allow correct positioning of the endoscope. To obtain reproducible results, the measurements were made in a fixed geometrical setup using a resection loop protruding exactly 12 mm from the front end of the endoscope and defining the distance from the tissue. Spectra (wavelength = 450–750 nm) were recorded from a central spot (2 mm in diameter) of the endoscopic fluorescence image and corrected for background, which was recorded before each series of measurements. Adequate blocking of excitation light was achieved by a combination of filters (KV430, GG455, Schott) located at the entrance slit of the spectrometer.

The spectra taken from the reference target enabled us to verify the correct adjustment of the optical multichannel analyzer.

Biopsies

Forty-five biopsies were taken during the course of the study. To allow correlation of fluorescence findings and histology, the macroscopic PPIX fluorescence for each biopsied site was classified as strong, weak, or negative.

For the assessment of sensitivity and specificity, histologic diagnoses of squamous cell carcinoma, carcinoma in situ and severe and moderate dysplasia were classified as malignant.

RESULTS

Fluorescence Examinations in 16 Patients

One to two hours after inhalation of 5-ALA aerosol, malignant lesions in the patient's larynx could be clearly distinguished from adjacent normal epithelium under fluorescence illumination. Whereas malignancies showed a strong red fluorescence, normal mucosa appeared in a greenish color when remitted excitation light was completely blocked with the OG515 filter (Fig. 1, middle). When some remitted excitation light was allowed (long pass = 440 nm), the appearance of normal tissue changed to a bluish color, and a significantly improved image quality was achieved due to the higher light intensity (Fig. 1, top and bottom).

However, in the case of strong bleeding, fluorescence detection was severely impeded due to the absorption characteristics of heme.

Evaluation of Fluorescence Spectra

Spectra taken from squamous cell carcinomas, carcinomas in situ, severe dysplasias, and corresponding normal tissues seemed to possess similar characteristics (Fig. 2). Whereas malignancies in general showed a higher porphyrin peak at 635 nm, the autofluorescence in the lower wavelength band (500–600 nm) was more intense in corresponding normal tissues.

To analyze the fluorescence contrast, tumor/normal (T/N) ratios were derived from spectra taken from malignancies and normal epithelium of two patients suffering from carcinoma in situ

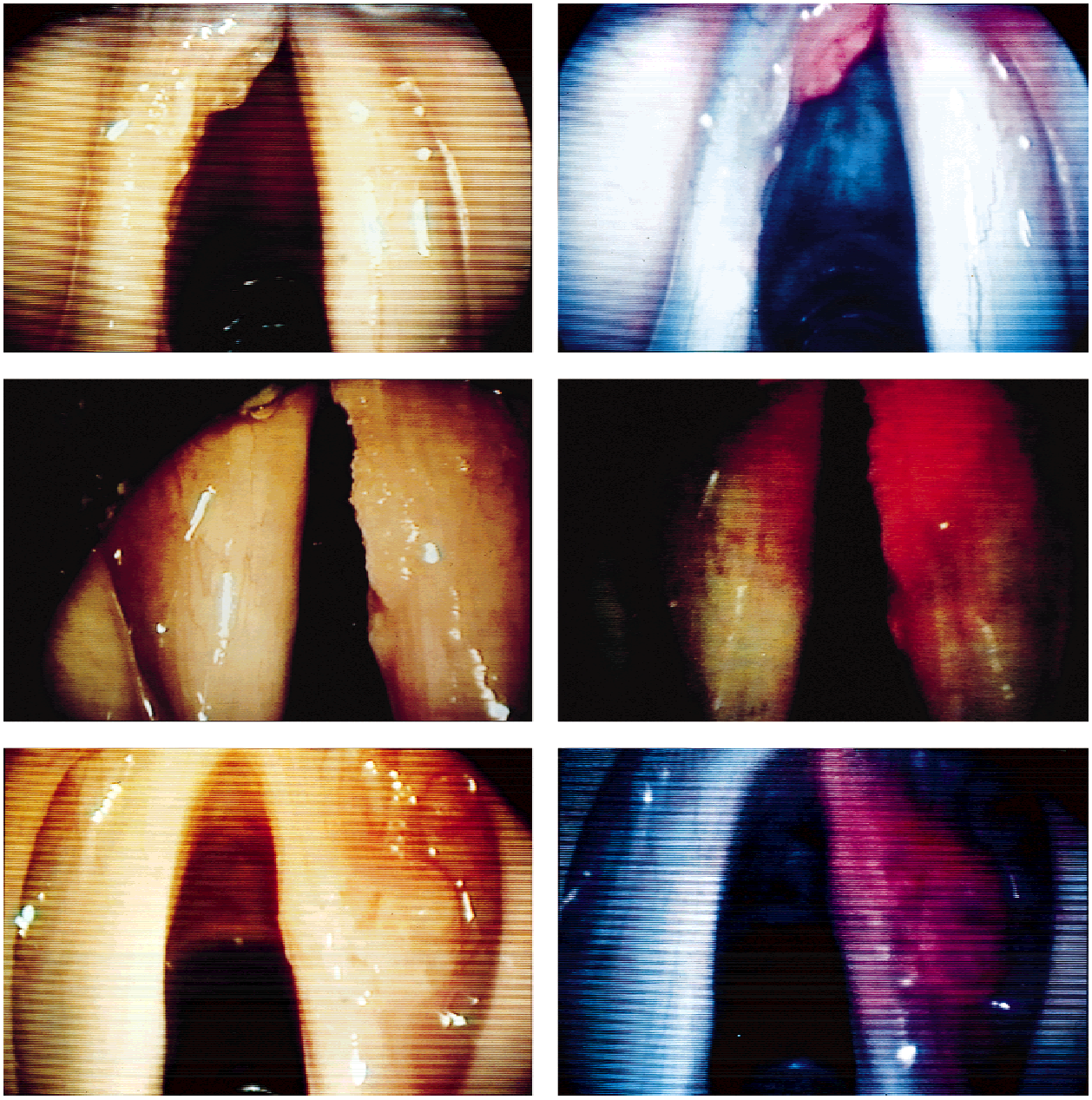

Fig. 1. White-light and fluorescence findings 2 hr after application of 5-aminolevulinic acid. **Top:** Moderate dysplasia of the left vocal cord under white light (left) and fluorescence illumination (right). **Middle:** Carcinoma in situ of the left vocal cord under white light (left) and fluorescence illumination (right). **Bottom:** Squamous cell carcinoma of the left vocal cord under white light (left) and fluorescence illumination (right).

and of six patients suffering from squamous cell carcinoma. The average T/N ratios computed for both groups are shown in Figure 3. A decrease (approximately 40–60%) of autofluorescence intensity of malignant tissue was observed in the spectral range of 500–600 nm. At 635 nm, the T/N ratio shows a value of almost 1.2–1.4, indicating that the intensity of PPIX fluorescence was on average 20–40% higher in tumorous tissue.

The average of T/N ratios normalized to autofluorescence (at 500 nm) showed a value of 3.6 at 635 nm for carcinoma in situ and 3.0 for squamous cell carcinoma.

Biopsies

The overall evaluation of biopsies obtained during the course of this study indicates (Table 1) that positive macroscopic PPIX fluorescence findings, which were classified as strong (F++), weak (F+), or negative (F–), were closely correlated to

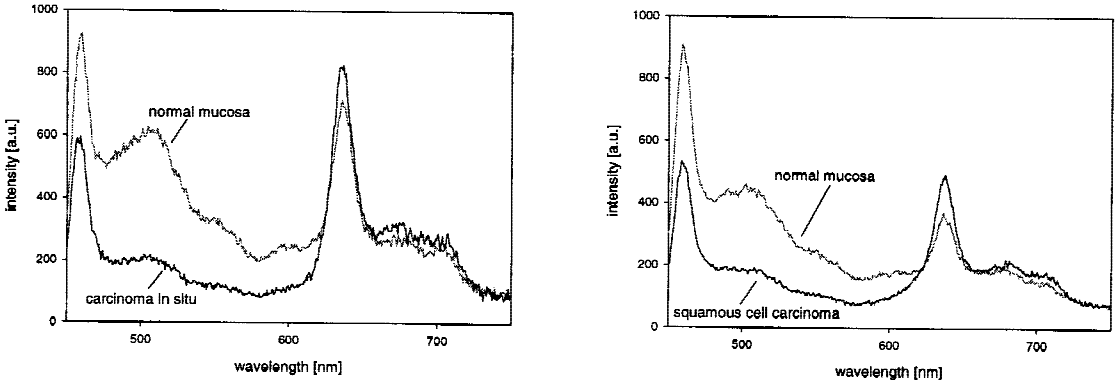

Fig. 2. **Left:** Average spectra of carcinoma in situ and corresponding normal tissue (n = 2 patients). **Right:** Average spectra of squamous cell carcinoma and corresponding normal tissue (n = 6 patients).

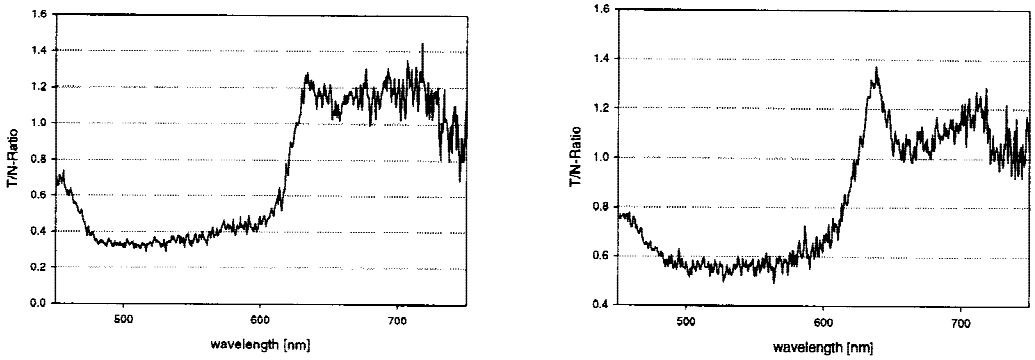

Fig. 3. Average T/N ratio of (left) carcinoma in situ (n = 2 patients) and (right) squamous cell carcinoma (n = 6 patients).

TABLE 1. Correlation of Macroscopic Fluorescence and Histology*

	PPIX Fluorescence Intensity		
Histology	F++	F+	F–
Normal epithelium	1	6	14
Mild dysplasia	4	—	—
Moderate dysplasia	1	—	1
Severe dysplasia	4	—	—
Carcinoma in situ	2	—	—
Squamous cell carcinoma	12	—	—

*PPIX, protoporphyrin IX; F++, strong; F+, weak; F–, negative.

malignant histologic findings. Of those 24 biopsies with a strong, macroscopically visible red fluorescence (F++), 19 were diagnosed to be squamous cell carcinoma, carcinoma in situ, and moderate or severe dysplasia, respectively. The weak fluorescence (F+) of six biopsies taken from normal epithelium could be macroscopically distinguished from those sites classified as strongly fluorescent (F++) and therefore was not counted as a false-positive result. Only one false-negative result, which was histologically diagnosed as moderate dysplasia, was observed.

According to these preliminary results, the sensitivity of this method was 95% and specifity was 80%.

DISCUSSION

Although there is a need for improving the diagnosis of laryngeal lesions, none of the techniques that have been developed for this purpose has found broad clinical acceptance.

Topical application of 5-ALA has been proven to selectively induce formation of PPIX in malignant tissues at various sites [15–17,19,20]. This effect has been attributed to changed enzyme activity patterns in malignant tissues and to an increased uptake of 5-ALA into malignant lesions due to a higher permeability of the surfaces of the lesions [22]. The strong red fluorescence, which originates from PPIX accumulation in malignancies, could easily be distinguished from the green autofluorescence signal of healthy tissues.

The inhalation method proposed in the present study avoids some of the obstacles experienced by the authors, who have been evaluating

developed methods for the detection of laryngeal cancer. For example, the surgeon is not required to perform any additional time-consuming procedures, such as the manual application of toluidine blue to the larynx, while the patient is under general anesthesia.

Initially observed variations in the quality of fluorescence staining resulting in false-positive or false-negative staining were most likely caused by variances in laryngeal aerosol deposition. Therefore, inhalation was performed at a rapid pace with intermittent vocalization, which has been reported to maximize total laryngeal aerosol deposition of an inhaled dose of aerosol [2,3].

Furthermore, the local application of 5-ALA and the rapid metabolism of the induced PPIX minimized unwanted photosensitization, which requires the patient to stay away from sunlight for several days after the systemic administration of HpD.

In contrast to the researchers using the various techniques based on autofluorescence [9,11] or tetracycline staining [14], the 5-ALA-induced fluorescence staining enabled us to clearly distinguish tumor from normal mucosa without high technical complexity. The appearance of malignant and normal tissue in different colors facilitates the detection of lesions. Thus, successful application of this technique becomes relatively independent on the examiner's experience, which has been reported as a problem with the autofluorescence-based approach.

The evaluation of spectra obtained during the course of this study confirms the macroscopic results and shows that the contrast between tumor and normal tissue originates from a selective accumulation of PPIX and from reduced autofluorescence intensities within the tumors. The average of T/N ratios, which was normalized to autofluorescence, approximately describes the visual impression and shows that only the combination of those two factors leads to a clearly visible contrast.

Because early stage malignancies, classified as carcinoma in situ, showed even higher T/N ratios when normalized to autofluorescence than did squamous cell carcinomas, the method seems especially well-suited for the detection of this early stage malignancy.

CONCLUSION

This study indicates a great potential for 5-ALA in the detection of early stage malignant laryngeal lesions. Because of its practicability and reliability, it may find the broad clinical acceptance that was denied alternative methods developed in the past.

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